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(71) Applicant *(for all designated States except US)*: **WYETH** [US/US]; 5 Giralda Farms, Madison, NJ 07940 (US).

(71) Applicants and
(72) Inventors: **O'TOOLE, Margot, Mary** [US/US]; 353 Albemarle Rd., Newton, MA 02460 (US). **LIU, Wei** [CN/US]; 73 Blackmer Road, Sudbury, MA 01776 (US).

(74) Agent: **VANDYKE, Raymond**; Nixon Peabody LLP, 401 9th Street N.W., Washington, DC 20004 (US).

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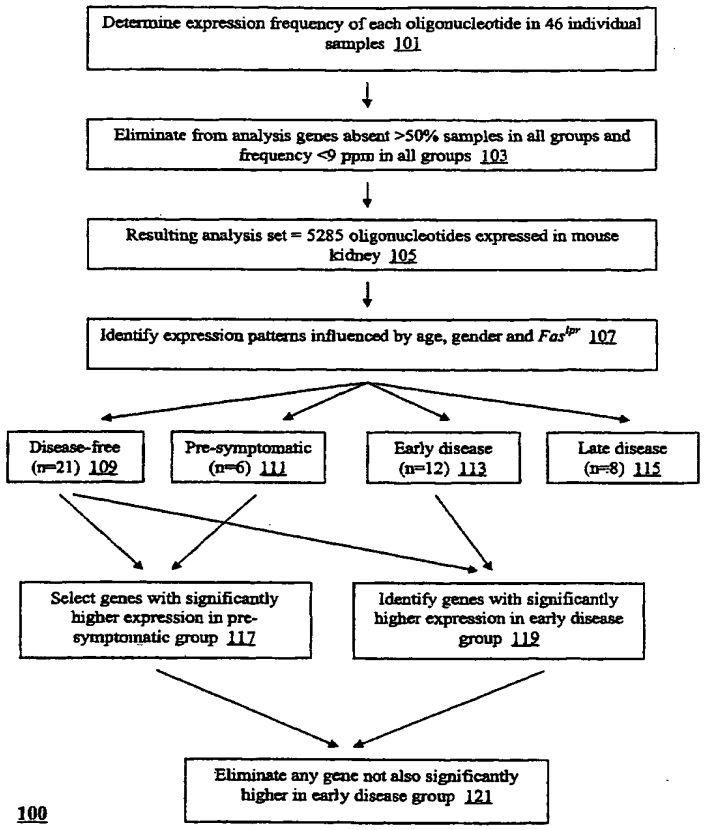
(54) Title: COMPOSITIONS AND METHODS FOR DIAGNOSING AND TREATING AUTOIMMUNE DISEASE




(57) Abstract: The present invention is generally directed to compositions and methods for the diagnosis, treatment, and prevention of lupus nephritis (LN), to the identification of novel therapeutic agents for LN, and to the creation of cell lines and animal models for studying the pathogenesis of the disease. The present invention is based on the discovery of transcribed polynucleotides that are either over-expressed or under-expressed in animals that develop lupus or are pre-disposed to lupus.



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# COMPOSITIONS AND METHODS FOR DIAGNOSING AND TREATING AUTOIMMUNE DISEASE

[0001] The present application incorporates by reference U.S. Provisional Application Serial No. 60/419,088, filed October 18, 2002 and entitled "Compositions and Methods for Diagnosing and Treating Autoimmune Disease."

## FIELD OF THE INVENTION

[0002] The present invention relates generally to diagnosis and treatment of autoimmune diseases. The invention specifically relates to diagnosing and treating systemic lupus erythematosus (SLE) and lupus nephritis (LN) by monitoring and modulating, respectively, midkine (MDK) activity or MDK gene expression.

## BACKGROUND

[0003] Lupus nephritis (LN) is an inflammation of the kidney caused by systemic lupus erythematosus (SLE). SLE, commonly known as lupus, is an autoimmune rheumatic disease characterized by deposition in tissues of autoantibodies and immune complexes leading to tissue injury. In contrast to autoimmune diseases such as multiple sclerosis and type 1 diabetes mellitus, SLE potentially involves multiple organ systems directly, and its clinical manifestations are diverse and variable. For example, some patients may demonstrate primarily skin rash and joint pain, show spontaneous remissions, and require little medication. At the other end of the spectrum are patients who demonstrate severe and progressive kidney involvement that requires immediate medical attention.

[0004] The serological hallmark of SLE, and the primary diagnostic test available until now, is elevated serum levels of IgG antibodies to constituents of the cell nucleus, such as double-stranded DNA (dsDNA), single-stranded DNA (ss-DNA), and chromatin. Among these autoantibodies, IgG anti-dsDNA antibodies play a major role in the development of LN. LN is a serious condition in which the capillary walls of the kidney's blood purifying glomeruli become thickened by accretions on the epithelial side of glomerular basement membranes. The disease is often chronic and progressive and may lead to eventual renal failure.

Females die at an average age of 17 weeks of age and males at 22 weeks. It has been demonstrated that the *Fas*$^{lpr}$ mutation is required for the development of LN and the subsequent death at an early age.

**[0010]** MRL/MpJ mice, the ancestral strain of MRL/MpJ-*Fas*$^{lpr}$, also exhibit autoimmune disorders but the symptoms are manifested much later in life compared to those of the MRL/MpJ-*Fas*$^{lpr}$ mice. Starting at about three months of age, levels of circulating immune complexes rise greatly in the MRL/MpJ-*Fas*$^{lpr}$ mouse but not in the wildtype control, MRL/MpJ. Also, beginning at 3 months MRL/MpJ-*Fas*$^{lpr}$ mice exhibit very severe proliferative glomerulonephritis, whereas in the MRL/MpJ controls usually only mild glomerular lesions are detected. The MRL/MpJ lymphoproliferation wild type females die at 73 weeks of age and males at 93 weeks, as in contrast to a lifespan of 17 weeks in the female and 22 weeks for males in the MRL/MpJ mouse homozygous for *Fas*$^{lpr}$. However, when the *Fas*$^{lpr}$ mutation is bred into other strains (C57BL/6 for example), kidney function remains normal through life. It thus appears that the MRL/MpJ mice have inherited a predisposition to developing lupus which is accelerated in the presence of the *Fas*$^{lpr}$ allele.

**[0011]** NZBxNZW F1 mouse is another animal model that develops an autoimmune disease resembling human SLE, with high titers of natural thymocyto-toxic autoantibody. NZBxNZW F1 hybrid B cells apparently differ from normal murine B cells in their capacity to produce IgG antibodies upon T cell-dependent antigenic stimulation. Genetic analysis of a backcross to NZW shows that one set of loci regulate serum levels of IgG antibodies to double-stranded DNA, single-stranded DNA, total histones and chromatin. These loci overlap with a second set of loci that control autoantibodies to the viral glycoprotein gp70. The second set of loci are most strongly linked with renal disease. A locus on distal chromosome 4 was linked with nephritis but not with any of the autoantibodies measured.

**[0012]** Treatment for SLE is directed at controlling the symptoms with the hope of putting the disease into remission. There are several chemotherapeutic agents in commercial use and available for remedial purposes. Most of these agents are not without side effects, some of which are severe and debilitating to the patient. Some non-steroidal anti-inflammatory agents may cause stomach upset and changes in kidney function, which can mimic some lupus symptoms themselves. Some anti-malarial drugs, when required at high dosage levels over a prolonged time frame, may accumulate in the retina and cause

[0017] The invention further provides cell lines harboring the MDK gene, animals transgenic for the MDK gene, and animals with an interrupted MDK gene (MDK knockout animals). These cell lines and animals can be used to study the functions of MDK.

[0018] In another embodiment, the present invention provides a method for diagnosing and monitoring SLE/LN by comparing the expression level of MDK at the nucleotide and/or protein level in biological samples from a subject to control samples.

[0019] In still another aspect, the invention provides polynucleotides capable of inhibiting MDK gene expression by RNA interference.

[0020] The invention further provides methods of inhibiting MDK gene expression by introducing siRNAs or other RNAi sequences into target cells.

[0021] The preferred embodiments of the inventions are described below in the Detailed Description of the Invention. Unless specifically noted, it is intended that the words and phrases in the specification and claims be given the ordinary and accustomed meaning to those of ordinary skill in the applicable art or arts. If any other meaning is intended, the specification will specifically state that a special meaning is being applied to a word or phrase.

[0022] It is further intended that the inventions not be limited only to the specific structure, material or methods that are described in the preferred embodiments, but in addition, include any and all structures, materials or methods that perform the claimed function, along with any and all known or later-developed equivalent structures, materials or methods for performing the claimed function.

[0023] Further examples exist throughout the disclosure, and it is not applicant's intention to exclude from the scope of the invention the use of structures, materials, or methods that are not expressly identified in the specification, but nonetheless are capable of performing a claimed function.

## BRIEF DESCRIPTION OF THE DRAWINGS

[0024] The inventions of this application are better understood in conjunction with the following drawings, in which:

[0025] FIGURE 1 is a flow chart describing the steps for selecting LN-related genes.

[0026] FIGURE 2 shows the gene expression frequency of MDK in LN-affected and control mice.

in the art, a polynucleotide/polypeptide can perform its desired function(s) even in the presence of considerable amounts of other components or molecules.

**[0033]** In some uses, a polynucleotide/polypeptide that is "substantially free of cellular material" includes preparations which have less than about 30% (by weight) other polynucleotides/polypeptides including contaminating polynucleotides/polypeptides. For instance, the preparations can have less than about 20%, less than about 10%, or less than about 5% other polynucleotides/polypeptides. If a polynucleotide/polypeptide preparation is recombinantly produced, it can be substantially free of culture medium, i.e., culture medium components representing less than about 20% by weight of the polynucleotide/polypeptide preparation.

**[0034]** The language "substantially free of chemical precursors" includes preparations in which the polynucleotide/polypeptide is separated from chemical precursors or other chemicals that are involved in the synthesis of the polynucleotide/polypeptide. In one embodiment, the language "substantially free of chemical precursors" includes kinase preparations having less than about 30% (by weight), less than about 20% (by weight), less than about 10% (by weight), or less than about 5% (by weight) chemical precursors or other chemicals used in the synthesis.

**[0035]** A "polynucleotide" can include any number of nucleotides.. For instance, a polynucleotide can have at least 20, 25, 30, 40, 50, 100 or more nucleotides. A polynucleotide can be DNA or RNA, double-stranded or single-stranded. A polynucleotide encodes a polypeptide if the polypeptide is capable of being transcribed and/or translated from the polynucleotide. Transcriptional and/or translational regulatory sequences, such as promoter and/or enhancer(s), can be added to the polynucleotide before said transcription and/or translation occurs. Moreover, if the polynucleotide is singled-stranded, the corresponding double-stranded DNA containing the original polynucleotide and its complementary sequence can be prepared before said transcription and/or translation.

**[0036]** As used herein, "a variant of a polynucleotide" refers to a polynucleotide that differs from the original polynucleotide by one or more substitutions, additions, and/or deletions. For instance, a variant of a polynucleotide can have 1, 2, 5, 10, 15, 20, 25 or more nucleotide substitutions, additions or deletions. Preferably, the modification(s) is in-frame, i.e., the modified polynucleotide can be transcribed and translated to the original or intended stop codon. If the original polynucleotide encodes a polypeptide with a

Table 1. Stringency Conditions

| Stringency Condition | Poly-nucleotide Hybrid | Hybrid Length (bp)[1] | Hybridization Temperature and Buffer[H] | Wash Temp. and Buffer[H] |
|---|---|---|---|---|
| A | DNA:DNA | >50 | 65°C; 1xSSC -or- 42°C; 1xSSC, 50% formamide | 65°C; 0.3xSSC |
| B | DNA:DNA | <50 | $T_B$*; 1xSSC | $T_B$*; 1xSSC |
| C | DNA:RNA | >50 | 67°C; 1xSSC -or- 45°C; 1xSSC, 50% formamide | 67°C; 0.3xSSC |
| D | DNA:RNA | <50 | $T_D$*; 1xSSC | $T_D$*; 1xSSC |
| E | RNA:RNA | >50 | 70°C; 1xSSC -or- 50°C; 1xSSC, 50% formamide | 70°C; 0.3xSSC |
| F | RNA:RNA | <50 | $T_F$*; 1xSSC | $T_f$*; 1xSSC |
| G | DNA:DNA | >50 | 65°C; 4xSSC -or- 42°C; 4xSSC, 50% formamide | 65°C; 1xSSC |
| H | DNA:DNA | <50 | $T_H$*; 4xSSC | $T_H$*; 4xSSC |
| I | DNA:RNA | >50 | 67°C; 4xSSC -or- 45°C; 4xSSC, 50% formamide | 67°C; 1xSSC |
| J | DNA:RNA | <50 | $T_J$*; 4xSSC | $T_J$*; 4xSSC |
| K | RNA:RNA | >50 | 70°C; 4xSSC -or- 50°C; 4xSSC, 50% formamide | 67°C; 1xSSC |
| L | RNA:RNA | <50 | $T_L$*; 2xSSC | $T_L$*; 2xSSC |
| M | DNA:DNA | >50 | 50°C; 4xSSC -or- 40°C; 6xSSC, 50% formamide | 50°C; 2xSSC |
| N | DNA:DNA | <50 | $T_N$*; 6xSSC | $T_N$*; 6xSSC |
| O | DNA:RNA | >50 | 55°C; 4xSSC -or- 42°C; 6xSSC, 50% formamide | 55°C; 2xSSC |
| P | DNA:RNA | <50 | $T_P$*; 6xSSC | $T_P$*; 6xSSC |
| Q | RNA:RNA | >50 | 60°C; 4xSSC -or- 45°C; 6xSSC, 50% formamide | 60°C; 2xSSC |
| R | RNA:RNA | <50 | $T_R$*; 4xSSC | $T_R$*; 4xSSC |

[1]: The hybrid length is that anticipated for the hybridized region(s) of the hybridizing polynucleotides. When hybridizing a polynucleotide to a target polynucleotide of unknown sequence, the hybrid length is assumed to be that of the hybridizing polynucleotide. When polynucleotides of known sequence are hybridized, the hybrid length can be determined by aligning the sequences of the polynucleotides and identifying the region or regions of optimal sequence complementarity.

[H]: SSPE (1xSSPE is 0.15M NaCl, 10mM $NaH_2PO_4$, and 1.25mM EDTA, pH 7.4) can be substituted for SSC (1xSSC is 0.15M NaCl and 15mM sodium citrate) in the hybridization and wash buffers.

$T_B$* - $T_R$*: The hybridization temperature for hybrids anticipated to be less than 50 base pairs in length should be 5-10°C less than the melting temperature ($T_m$) of the hybrid, where $T_m$ is determined according to the following equations. For hybrids less than 18 base pairs in length, $T_m$(°C) = 2(# of A + T bases) + 4(# of G + C bases). For hybrids

glutamine, or serine, threonine, phenylalanine and tyrosine. Other groups of amino acids that can produce conservative changes include: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. A polypeptide variant can also contain nonconservative changes.

**[0044]** Polypeptide variants can be prepared by substituting, modifying, deleting and/or adding one or more amino acids that have minimal influence on the biological activity, immunogenicity, secondary structure and/or hydropathic nature of the polypeptide. Variants can be prepared by substituting, deleting or adding, for example, 1, 2, 5, or 10 amino acids residues in the original sequence. Polypeptide variants preferably exhibit at least about 70%, more preferably at least about 90%, and most preferably at least about 95% sequence homology to the original polypeptide.

**[0045]** Polypeptide variants include polypeptides that are modified from the original polypeptides either by a natural process, such as a post-translational modification, or by a chemical modification. These modifications are well known in the art. Modifications can occur anywhere in the polypeptide, including the backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification can be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide can contain many types of modifications. Polypeptides may be branched, for example, as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched, and branched cyclic polypeptides can result from natural post-translational processes or be made through synthetic methods. Suitable modifications for this invention include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, pegylation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination.

[0053] Various aspects of the invention are described in further detail in the following subsections. The use of subsections is not meant to limit the invention, but rather the subsections may apply to any aspect of the invention.

*Midkine and LN*

[0054] The gene expression pattern in kidneys of 4 different strains of mice: MRL/MpJ-*Fas*$^{lpr}$, MRL/MpJ, C57Bl6 and C57Bl6/*Fas*$^{lpr}$, were evaluated using the Affymetrix Mu11KsubA and Mu11KsubB oligonucleotide arrays (Affymetrix, Santa Clara, CA).

[0055] Briefly, the gene expression analysis was performed using kidney RNA samples harvested from individual mice. The RNA samples were reverse transcribed into cDNA and hybridized to the oligonucleotide arrays. The results were analyzed using Microarray Suite software. A gene analysis set of 5285 oligonucleotides was first selected using the criteria described in Example 3. The expression frequency of each gene on the 5285 oligonucleotides in the gene analysis set was then determined for all C57Bl6, C57Bl6/*Fas*$^{lpr}$, MRL/MpJ-*Fas*$^{lpr}$ and MRL/MpJ kidney samples (n = 46). In order to identify gene expression patterns that may contribute disease initiation, selected first were genes with significantly different expression levels in young, pre-symptomatic MRL/MpJ kidney and kidneys from mice that do not develop LN. Late stage disease samples (*i.e*, samples from MRL/MpJ-*Fas*$^{lpr}$ mice four months of age or older) were omitted from this initial screen due to the numerous and profound changes in gene expression related to inflammation, kidney failure and fibrosis observed at this stage of disease. These changes are known consequences of the disease process, and would be expected to obscure differences identified between disease free and early stage disease samples.

[0056] Figure 1 shows a flow chart describing the process 100 for selecting LN-related genes. Defining significant difference between groups as $p < 0.0005$ (two tailed student t test, unequal variance) and average fold change (AFC) >1.5, a list of genes with significant expression frequency differences between lupus nephritis negative samples (C57BL/6, C57BL6/Fas$^{lpr}$) and young (pre-symptomatic) MRL/MpJ-*Fas*$^{lpr}$ kidneys was compiled (step 101). Genes on this list that did not also show significant expression level differences (again defined as $p<0.0005$, AFC >1.5) between lupus nephritis negative samples and early stage disease samples (*i.e.*, samples consisting of the 6 older MRL/MpJ and the 6 young MRL/MpJ-*Fas*$^{lpr}$ samples) were removed from the list (step 103). This

*(1) Function in neurone-glial interaction*

**[0060]** MDK expression during development is temporally and spatially regulated. The patterns of expression suggest that MDK plays a role in neural maturation, epithelial/mesenchyme interactions and secondary embryonic induction processes. Generally, MDK expression in the central nerve system (CNS) occurs early in embryogenesis and is completed by birth. MDK expression is barely detectable in adults except in kidney and certain CNS areas. Immunohistochemical studies revealed MDK to be localized beside radial glial processes along which neurons migrate. It has been suggested that MDK is synthesized by and localized on the surface of radial glial cells, while its receptor system is localized on neurons (Sun *et al.,* J. Neuropathol Exp Neurol 56:1339-1348, 1997). Upon experimental infraction in rats, MDK expression in the isochemic brain begins as early as 1 day after the operation. MDK expression is also induced in Alzheimer's senile plaque and in photoreceptor cells rescued from light-induced damage, suggesting involvement of MDK in repair and regeneration mechanisms in the nervous system. MDK has also been reported to promote neuronal survival in culture, to stimulate neuronal differentiation, and to be involved in synaptogenesis. As MDK is expressed in glial cells, most notably in the radial glia of the neocortex and the Bergmann fibres of the cerebellar cortex, it may also have a role in the migration of neuron progenitors prior to the onset of axon navigation.

*(2) Function in inflammatory responses*

**[0061]** MDK has also been detected in synovial fluid, synoviocytes, and endothelial cells of new blood vessels in the inflammatory synovitis of rheumatoid arthritis and osteoarthritis, but was not detected in normal synovial fluid and non-inflammatory synovial tissues. MDK promotes chemotaxis of neutrophils and histamine release from rat peritoneal mast cells in a dose-dependent manner. MDK also enhances plasminogen activator activity and reduces plasminogen activator inhibitor levels in bovine aortic endothelial cells. These activities of MDK are in agreement with the modes of MDK expression in various pathological states. It has thus been suggested that MDK is an important molecule regulating inflammatory responses.

(3) *Function in tumorigenesis and angiogenesis*

MDK has been found to suppress tumorigenicity of rectal carcinoma cells in nude mice (Takei *et al.*, Cancer Res 61:8486-8491, 2001; Takei *et al.*, J Biol Chem 2002).

*(4) MDK's involvement in signal pathways*

**[0065]** Ligand homodimerization appears to be the initial step in the activation of a common signal pathway for MDK. Dimer formation through transglutaminase-mediated cross-linking is important for the biological activity of MDK. Proteoglycan-binding enhances the biological activities of MDK in a manner analogous to members of the fibroblast growth factor family. Covalently bound homodimers are uncommon amongst tyrosine kinase receptor ligands. In the case of MDK, there is evidence that stable non-disulphide bonded dimers are formed through the catalytic activity of type-2 transglutaminase, an enzyme which crosslinks neural substrates through ε-(γ-glutamyl)lysine isopeptide bonds. Upon incubation with transglutaminase, MDK forms multimers through cross-linkages. It was found that (1) heparin potentiated the multimer formation; (2) the N- and C-terminal half domains each formed a dimer through the action of transglutaminase; (3) Gln42 or Gln44 in the N-terminal half and Gln95 in the C-terminal half served as amine acceptors in the cross-linking reaction; and (4) MDK-derived peptide Ala41-Pro51 strongly inhibited the cross-linking and abolished the biological activity of MDK to enhance the plasminogen activator activity in bovine aortic endothelial cells. The inhibition, however, was limited against the MDK monomer and was not seen against the MDK dimer, suggesting that dimer formation through transglutaminase-mediated cross-linking is an important step affecting the biological activity of MDK. This notion is further supported by the finding that the interaction of dimeric MDK with corresponding glycosaminoglycan binding sites is the basis for cooperation of proteoglycans in signaling. Since the isopeptide bond is highly resistant to degradation, this novel means of covalent association potentiates signaling.

**[0066]** It is likely that two or more high-affinity tyrosine kinase receptors exist for MDK. MDK forms dimers before associating with its receptors and appears to activate tyrosine kinase, JAK/STAT-1 and PI 3-kinase signal pathways. These properties suggest that MDK is a ligand for tyrosine kinase receptors. MDK also interacts strongly with cell surfaces, and its binding sites include proteoglycans. Studies to date on the signal pathways of MDK suggest that its common cell surface binding domains in neurons have at least three interactive components: syndecan-3/proteoglycan complexes, the receptor-

rescued G401 cells from CDDP-induced apoptosis. It was found that MDK enhanced the expression of Bcl-2, but not that of Bcl-x(L), in G401 cells in a dose-dependent manner. MDK also prevented the Bcl-2 reduction due to CDDP. Moreover, Bcl-2 expression in mouse kidney was also transiently suppressed by CDDP treatment, the expression profile being similar to that of MDK. It thus appears that MDK exerts cytoprotective activity toward a damaging insult, presumably at least in part through enhancement of the expression of Bcl-2.

*(6) MDK protein structure*

**[0070]** Human MDK is a secreted glycoprotein with a molecular mass of about 13 kD. Human MDK precursor has 143 amino acid residues (SEQ ID NO:1), including a 22-amino acid leader peptide (SEQ ID NO:2). Mature MDK (SEQ ID NO:3) is structurally divided into two domains, an N-terminal domain and a C-terminal domain. The solution structure of the two domains was determined by NMR (Iwasake *et al.*, EMBO J 16:6936-6946, 1997). Both domains consist of three antiparallel beta-sheets, but the C-terminal domain has a long flexible hairpin loop where a heparin-binding consensus sequence is located. Basic residues on the beta-sheet of the C-terminal domain form another heparin-binding site. Measurement of NMR signals in the presence of heparin oligosaccharides verified that multiple amino acids in the two sites participated in heparin binding.

**[0071]** A 121-amino-acid-residue human MDK (SEQ ID NO:3) has been synthesized in solution (Peptide Institute Inc., J Pept Sci 2:28-39, 1996). The final product was confirmed to have the correct disulphide structure from its tryptic peptide mapping and to possess the same biological activities as those of the natural product. The N- and C-terminal domains [(1-59aa) and (60-121aa), respectively] were also synthesized. The C-terminal domain showed the full pattern of bioactivities except for the neuronal cell survival activity, while the N-terminal domain had much less activity in general (supra). The 13 amino acid residues in the C-terminal end were found to be responsible for the MDK antigenicity (Muramatsu *et al.*, Biochem Biophys Res Commun 203:1131-1139, 1994).

**[0072]** MDK is also characterized by a high content (*e.g.*, about 25%) of basic amino acid residues, predominately lysine, which result in the proteins having high pI values of around 10. The basic residues are not evenly distributed throughout the polypeptide chain, but are clustered at the N-terminus and at the C-terminus with another concentration in the

**[0074]** The inhibitory activities of various heparin derivatives toward interaction of MDK with neurons were also examined (Kaneda *et al.*, Biochem Biophys Res Commun 220:108-112, 1996). All of the three sulfate groups in the heparin disaccharide unit (6-O-sulfate, 2-O-sulfate and N-sulfate) were necessary for full inhibitory activity. Among these, the N-sulfate group was critically important. The minimum size with inhibitory activity was approximately 7 kd. Thus, the highly sulfated region in cell surface heparin sulfate proteoglycan is required for neurons to interact with MDK.

*(7) MDK gene and MDK promoter*

**[0075]** Human MDK gene (SEQ ID NO:4) contains five exons and four introns with the coding sequences present in exons 2-5. The MDK gene is located on chromosome 11q11.2. MDK gene sequences from different species share a high level of homology. For example, human and mouse MDK is 87% identical at amino acid level and most amino acid changes are conservative. All the characteristic cysteine and lysine residues are conserved. This high degree of evolutional conservation reinforces the important role of MDK during embryogenesis. The organization of the human MDK gene is also similar to that of the mouse MDK gene. All exon-intron boundaries are conserved between mouse and human MDK. It was found that a 170 base block in the upstream region of the putative transcription initiation sites and three blocks of 200-350 bases in regions further upstream are highly conserved. These homologous blocks may play important roles in developmentally-regulated expression of the MDK gene. Further analysis revealed that the 2.3 kb upsteam sequence of the human MDK gene has cis-acting elements which confer retinoic acid-induced expression of a fused chloramphenicol acetyl-transferase (CAT) gene in F9 embryonal carcinoma cells. In the 5'-region of the human MDK gene, a sequence resembling the DR5-type retinoic acid-responsive element (*i.e.*, AGGTCA-related direct repeats separated by 5 nucleotides) is present in a small block that is highly homologous between the human and mouse genes. Deletion of this direct repeat reduced retinoic acid-induced CAT gene expression (Pedraza *et al.*, J Biochem 117:845-849, 1995). Because the MDK promoter appears to be active only in tumor tissues, the human MDK promoter has been used in adenoviral suicide gene therapy for pancreatic cancer and MDK-positive pediatric tumor.

**[0076]** Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis revealed an association of an intronic polymorphism in the MDK gene

biochips, such as the GeneChip®, as probes to detect MDK mRNA. Anti-MDK antibody can be developed and used in diagnostic kits to detect MDK protein levels in body fluids. The markers can be used to provide diagnosis or prognosis information in a particular subject sample or to assess the efficacy of a treatment or therapy of SLE/LN. For example, comparison of expression levels of MDK at different stages of the disease progression provides a method for long-term prognosing, including survival. MDK gene polymorphism may also be indicative to a subject's susceptibility to SLE/LN. In another example, the evaluation of a particular treatment regime may be evaluated, including whether a particular drug will act to improve the long-term prognosis in a particular patient.

**[0081]** MDK promoter, MDK gene, and MDK gene products (the transcribed polynucleotides and the translated polypeptides) can be targets for a treatment or therapeutic agent. They can also be used to generate gene therapy vectors that inhibit lupus.

**[0082]** Therefore, without limitation as to mechanism, the present invention is based in part on the principle that modulation of the expression of the MDK gene expression may ameliorate SLE/LN, when they are expressed at levels similar or substantially similar to normal (non-diseased) tissue. The modulation may occur at transcriptional, post-transcriptional, translational, and post-translational levels. For example, MDK promoter may be targeted to inhibit transcription. MDK mRNA may be targeted by anti-sense molecules to prevent translation. The post-translational processing of MDK protein, such as leader peptide removal, glycosylation and dimerization, may also be targeted.

**[0083]** The discovery of the MDK gene expression patterns in SLE/LN affected animal allows for screening of test agents with the goal of modulating MDK expression or MDK activity. The test agents may be screened by their effect on MDK expression at mRNA or protein level, or by their effect on the activity of MDK.

**[0084]** In another embodiment of the invention, a modulator of MDK expression or MDK activity may be used as a therapeutic agent for SLE and LN. The modulator may be a polynucleotide such as an antisense oligonucleotide, a polypeptide such as an anti-MDK antibody or a MDK mutant having a dominant negative effect on a activity of the wild-type MDK, a viral or non-viral gene therapy vector, or any other organic or inorganic molecule that is capable of inhibiting MDK activity or MDK expression. Formulation of such modulator into pharmaceutical compositions is described in subsections below.

more preferably about 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 400 or more consecutive nucleotides of a MDK gene, or a polynucleotide transcribed thereof.

**[0090]** Probes based on the nucleotide sequence of a MDK gene, or a polynucleotide transcribed thereof can be used to detect transcripts or genomic sequences corresponding to the MDK gene, or a polynucleotide transcribed thereof. In preferred embodiments, the probe comprises a label group attached thereto, *e.g.*, the label group can be a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. Such probes can be used as a part of a diagnostic test kit for identifying cells or tissue which mis-express (*e.g.*, over- or under-express) a MDK gene, or which have greater or fewer copies of a MDK gene. For example, a level of a MDK gene product in a sample of cells from a subject may be detected, the amount of mRNA transcript of MDK may be determined, or the presence of mutations or deletions of a MDK gene may be assessed.

**[0091]** The invention also specifically encompasses homologs of the MDK gene of other species. Gene homologs are well understood in the art and are available using databases or search engines such as the Pubmed-Entrez database.

**[0092]** The invention also encompasses polynucleotides that are structurally different from the molecules described above (*i.e.*, which have a slight altered sequence), but which have substantially the same properties as those above (*e.g.*, encoded amino acid sequences, or which are changed only in non-essential amino acid residues). Such molecules include allelic variants, and are described in greater detail in subsections herein.

**[0093]** In addition to the nucleotide sequences of the MDK gene, it will be appreciated by those of skill in the art that DNA sequence polymorphisms that lead to changes in the amino acid sequences of the proteins encoded by the MDK gene may exist within a population (*e.g.*, the human population). Such genetic polymorphism in the MDK gene may exist among individuals within a population due to natural allelic variation. An allele is one of a group of genes which occur alternatively at a given genetic locus. In addition it will be appreciated that DNA polymorphisms that affect RNA expression levels can also exist that may affect the overall expression level of that gene (*e.g.*, by affecting regulation or degradation). As used herein, the phrase "allelic variant" includes a nucleotide sequence which occurs at a given locus or to a polypeptide encoded by the nucleotide sequence.

**[0094]** Polynucleotides corresponding to natural allelic variants and homologs of the MDK gene can be isolated based on their homology to the human MDK gene, using the

standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. Alternatively, mutations can be introduced randomly along all or part of a coding sequence of the MDK gene or cDNA, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity to identify mutants that retain activity. Following mutagenesis, the encoded protein can be expressed recombinantly and the activity of the protein can be determined.

**[0099]** In yet another aspect of the invention, a polynucleotide may encode a MDK protein containing mutations in amino acid residues which result in inhibition of MDK activity after dimerization with a wild-type MDK protein. These mutated MDK proteins can be used to inhibit MDK activity in a SLE/LN patient.

**[0100]** A polynucleotide of this invention can be further modified to increase stability *in vivo*. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends; the use of phosphorothioate or 2-o-methyl rather than phosphodiester linkages in the backbone; and/or the inclusion of nontraditional bases such as inosine, queosine and wybutosine, as well as acetyl- methyl-, thio- and other modified forms of adenine, cytidine, guanine, thymine and uridine.

**[0101]** Another aspect of the invention pertains to isolated polynucleotides, which are antisense to a MDK gene. An "antisense" polynucleotide comprises a nucleotide sequence which is complementary to a "sense" polynucleotide encoding a protein, *e.g.*, complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Accordingly, an antisense polynucleotide can bind via hydrogen bonds to a sense polynucleotide. The antisense polynucleotide can be complementary to an entire coding strand of a gene of the invention or to only a portion thereof. In one embodiment, an antisense polynucleotide is antisense to a "coding region" of the coding strand of a nucleotide sequence of the invention. The term "coding region" includes the region of the nucleotide sequence comprising codons which are translated into amino acid. In another embodiment, the antisense polynucleotide is antisense to a "noncoding region" of the coding strand of a nucleotide sequence of the invention.

**[0102]** Antisense polynucleotides of the invention can be designed according to the rules of Watson and Crick base pairing. The antisense polynucleotide can be complementary to the entire coding region of an mRNA corresponding to a gene of the invention, but more preferably is an oligonucleotide which is antisense to only a portion of

polynucleotides of the invention is direct injection at a tissue site (*e.g.*, intestine or blood). Alternatively, antisense polynucleotides can be modified to target selected cells and then administered systemically. For example, for systemic administration, antisense molecules can be modified such that they specifically bind to receptors or antigens expressed on a selected cell surface, *e.g.*, by linking the antisense polynucleotides to peptides or antibodies which bind to cell surface receptors or antigens. The antisense polynucleotides can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense polynucleotides is placed under the control of a strong pol II or pol III promoter are preferred.

**[0104]** In yet another embodiment, the antisense polynucleotide of the invention is an α-anomeric polynucleotide. An α-anomeric polynucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other. The antisense polynucleotide can also comprise a 2'-o-methylribonucleotide or a chimeric RNA-DNA analogue.

**[0105]** In still another embodiment, an antisense polynucleotide is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded polynucleotide, such as an mRNA, to which they have a complementary region. Thus, ribozymes can be used to catalytically cleave mRNA transcripts of MDK to thereby inhibit translation of said mRNA. A ribozyme having specificity for a MDK polynucleotide can be designed based upon the nucleotide sequence of a MDK gene. An mRNA transcribed from a MDK gene can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules.

**[0106]** Alternatively, expression of a MDK gene can be inhibited by targeting nucleotide sequences complementary to the regulatory region of these genes (*e.g.*, the promoter and/or enhancers) to form triple helical structures that prevent transcription of the gene in target cells.

**[0107]** Expression of MDK gene can also be inhibited using RNA interference ("RNAi"). RNAi is a phenomenon in which the introduction of double-stranded RNA (dsRNA) into certain organisms or cell types causes degradation of the homologous mRNA. First discovered in the nematode *Caenorhabditis elegans*, RNAi has since been found to operate in a wide range of organisms. For example, in mammalian cells, introduction of long dsRNA (>30 nucleotides) can initiate a potent antiviral response,

specific gene silencing can be achieved in mammalian cells. These siRNAs can specifically suppress targeted gene translation in mammalian cells without activation of DNA-dependent protein kinase (PKR) by longer dsRNA, which may result in non-specific repression of translation of many proteins.

**[0111]** Second, siRNAs can be expressed *in vivo* from vectors. This approach can be used to stably express siRNAs in cells or transgenic animals. In one embodiment, siRNA expression vectors are engineered to drive siRNA transcription from polymerase III (pol III) transcription units. Pol III transcription units are suitable for hairpin siRNA expression, since they deploy a short AT rich transcription termination site that leads to the addition of 2 bp overhangs (*e.g.*, UU) to hairpin siRNAs - a feature that is helpful for siRNA function. The Pol III expression vectors can also be used to create transgenic mice that express siRNA.

**[0112]** In another embodiment, siRNAs can be expressed in a tissue-specific manner. Under this approach, long double-stranded RNAs (dsRNAs) are first expressed from a promoter (such as CMV (pol II)) in the nuclei of selected cell lines or transgenic mice. The long dsRNAs are processed into siRNAs in the nuclei (*e.g.*, by Dicer). The siRNAs exit from the nuclei and mediate gene-specific silencing. A similar approach can be used in conjunction with tissue-specific promoters to create tissue-specific knockdown mice.

**[0113]** Any 3' dinucleotide overhang, such as UU, can be used for siRNA design. In some cases, G residues in the overhang are avoided because of the potential for the siRNA to be cleaved by RNase at single-stranded G residues.

**[0114]** With regard to the siRNA sequence itself, it has been found that siRNAs with 30–50% GC content can be more active than those with a higher G/C content in certain cases. Moreover, since a 4–6 nucleotide poly(T) tract may act as a termination signal for RNA pol III, stretches of $\geq$ 4 Ts or As in the target sequence may be avoided in certain cases when designing sequences to be expressed from an RNA pol III promoter. In addition, some regions of mRNA may be either highly structured or bound by regulatory proteins. Thus, it may be helpful to select siRNA target sites at different positions along the length of the gene sequence. Finally, the potential target sites can be compared to the appropriate genome database (human, mouse, rat, etc.). Any target sequences with more than 16–17 contiguous base pairs of homology to other coding sequences may be eliminated from consideration in certain cases.

remaining 19-mer sequence does not include any four consecutive A or T (i.e., AAAA or TTTT) or seven "GC" in a role. Exemplary RNAi target sequences identified according to the above-described criteria are illustrated in Table 2. The siRNA sequence for each target sequence (the sense strand and the antisense strand), and the 5' end location of each target sequence in SEQ ID NO:4 ("5 End") are also indicated in Table 2.

**[0121]** Additional criteria can also be used for RNAi target sequence design. For instance, the GC content of the remaining 19-mer sequence can be limited to between 45% and 55%. Moreover, any 19-mer sequence having three consecutive identical bases (i.e., GGG, CCC, TTT, or AAA) or a palindrome sequence with 5 or more bases is excluded. Furthermore, the remaining 19-mer sequence can be selected to have low sequence homology to other human genes. In one specific example, potential target sequences are searched by BLASTN against NCBI's human UniGene cluster sequence database. The human UniGene database contains non-redundant sets of gene-oriented clusters. Each UniGene cluster includes sequences that represent a unique gene. 19-mer sequences producing no hit to other human genes under the BLASTN search can be selected. During the search, the e-value may be set at a stringent value (such as "1").

| Target Sequence (SEQ ID NO) | 5' End | siRNA Sense Strand (SEQ ID NO) | siRNA Antisense Strand (SEQ ID NO) |
|---|---|---|---|
| GAGCCGACTGCAAGTACAAGT (SEQ ID NO:44) | 266 | GCCGACUGCAAGUACAAGUUU (SEQ ID NO:45) | UUCGGCUGACGUUCAUGUUCA (SEQ ID NO:46) |
| GACTGCAAGTACAAGTTTGAG (SEQ ID NO:47) | 271 | CUGCAAGUACAAGUUUGAGUU (SEQ ID NO:48) | UUGACGUUCAUGUUCAAACUC (SEQ ID NO:49) |
| GACCAAAGCAAAGGCCAAAGC (SEQ ID NO:50) | 411 | CCAAAGCAAAGGCCAAAGCUU (SEQ ID NO:51) | UUGGUUUCGUUUCCGGUUUCG (SEQ ID NO:52) |

Alternatively, chimeric molecules can be synthesized with a 5' DNA segment and a 3' PNA segment.

**[0125]** In other embodiments, the oligonucleotide may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane or the blood-kidney barrier. In addition, oligonucleotides can be modified using hybridization-triggered cleavage agents or intercalating agents. To this end, the oligonucleotide may be conjugated to another compound (*e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, or hybridization-triggered cleavage agent). Finally, the oligonucleotide may be detectably labeled, either such that the label is detected by the addition of another reagent (*e.g.*, a substrate for an enzymatic label), or is detectable immediately upon hybridization of the nucleotide (*e.g.*, a radioactive label or a fluorescent label).

***Polypeptides and Variants Thereof***

**[0126]** Several aspects of the invention pertain to isolated MDK polypeptides and mutated MDK polypeptides capable of inhibiting normal MDK activity. The present invention also contemplates immunogenic polypeptide fragements suitable for raising anti-MDK antibodies.

**[0127]** In one embodiment, native MDK polypeptides can be isolated from cells or tissue sources by an appropriate purification scheme using standard protein purification techniques. Standard purification methods include electrophoretic, molecular, immunological and chromatographic techniques, including ion exchange, hydrophobic, affinity, and reverse-phase HPLC chromatography, and chromatofocusing. For example, the MDK polypeptides may be purified using a standard anti-MDK antibody column. Ultrafiltration and diafiltration techniques can also be used. The degree of purification necessary depends on the purpose of the MDK polypeptides. In some instances purification will not be necessary.

**[0128]** In another embodiment, MDK polypeptides or mutated MDK polypeptides capable of inhibiting normal MDK activity (dominant-negative mutants) are produced by recombinant DNA techniques. Alternative to recombinant expression, MDK polypeptides or mutated MDK polypeptides can be synthesized chemically using standard peptide synthesis techniques.

[0132] The invention also provides chimeric or fusion MDK polypeptides. A fusion MDK polypeptide contains a MDK-related polypeptide and a non-MDK polypeptide fused in-frame to each other. The MDK-related polypeptide corresponds to all or a portion of a MDK polypeptide or its variant. In a preferred embodiment, a fusion MDK polypeptide comprises at least one portion of a MDK polypeptide sequence recited in SEQ ID NO:1.

[0133] A peptide linker sequence may be employed to separate the MDK-related polypeptide from non-MDK polypeptide components by a distance sufficient to ensure that each polypeptide folds into its secondary and tertiary structures. Such a peptide linker sequence is incorporated into the fusion protein using standard techniques well known in the art. Suitable peptide linker sequences may be chosen based on the following factors: (1) their ability to adopt a flexible extended conformation; (2) their inability to adopt a secondary structure that could interact with functional epitopes on the MDK-related polypeptide and non-MDK polypeptide; and (3) the lack of hydrophobic or charged residues that might react with the polypeptide functional epitopes. Preferred peptide linker sequences contain Gly, Asn and Ser residues. Other near neutral amino acids, such as Thr and Ala may also be used in the linker sequence. Amino acid sequences suitable as linkers include those disclosed in Maratea *et al.*, Gene 40:39-46, 1985; Murphy *et al.*, Proc. Natl. Acad. Sci. USA 83:8258-8262, 1986; U.S. Pat. No. 4,935,233 and U.S. Pat. No. 4,751,180. The linker sequences may generally be from about 1 to about 50 amino acids in length. Linker sequences are not required when the MDK-related polypeptide or the non-MDK polypeptide have non-essential N-terminal amino acid regions that can be used to separate the respective functional domains and thereby prevent steric interference.

[0134] For example, in one embodiment, the fusion protein is a GST-MDK fusion protein in which the MDK-related sequences are fused to the C-terminus of the GST sequences. Such fusion proteins can facilitate the purification of recombinant MDKs.

[0135] The MDK-fusion proteins of the invention can be incorporated into pharmaceutical compositions and administered to a subject *in vivo*, as described herein. The MDK-fusion proteins can be used to affect the bioavailability of a MDK substrate. Use of MDK-fusion proteins may be useful therapeutically for the treatment of or prevention of damage caused by, for example, (i) aberrant modification or mutation of MDK, and (ii) aberrant post-translational modification of MDK. It is also conceivable that a fusion protein containing a normal or mutated MDK polypeptide, or a fragment thereof may be capable of inhibiting MDK activity in a subject.

signal sequence is subsequently or concurrently cleaved. The protein can then be readily purified from the extracellular medium by art recognized methods.

**[0140]** Alternatively, the signal sequence can be linked to the protein of interest using a sequence which facilitates purification, such as with a GST domain.

**[0141]** The present invention also pertains to variants of MDK which function as antagonists to MDK. In one embodiment, antagonists or agonists of MDK are used as therapeutic agents. For example, antagonists to MDK that can decrease the activity or expression of MDK may ameliorate SLE/LN in a subject wherein MDK is abnormally increased in level or activity. Variants of MDKs can be generated by mutagenesis, *e.g.*, discrete point mutation or truncation of a MDK.

**[0142]** In certain embodiments, an antagonist of a MDK can inhibit one or more of the activities of the naturally occurring form of the MDK by, for example, competitively modulating an activity of the MDK. Thus, specific biological effects can be elicited by treatment with a variant of limited function.

**[0143]** Mutants of a MDK which function as either MDK agonists or as MDK antagonists can be identified by screening combinatorial libraries of mutants. In certain embodiments, such variants may be used for example as a therapeutic protein of the invention. A variegated library of MDK variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential MDK sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (*e.g.*, for phage display) containing the set of MDK sequences therein. There are a variety of methods which can be used to produce libraries of potential MDK variants from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be performed in an automatic DNA synthesizer, and the synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one mixture, of all of the sequences encoding the desired set of potential MDK sequences. Methods for synthesizing degenerate oligonucleotides are known in the art.

**[0144]** In addition, libraries of fragments of a protein coding sequence corresponding to a MDK can be used to generate a variegated population of MDK fragments for screening and subsequent selection of variants of a MDK. In one embodiment, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of a MDK coding sequence with a nuclease under conditions wherein nicking occurs only

an antigen if the binding affinity between the antibody and the antigen is equal to or greater than $10^5$ $M^{-1}$. The antibodies can be monoclonal or polyclonal. Preferably, the antibodies are monoclonal. More preferably, the antibodies are humanized antibodies.

**[0149]** In another aspect, the invention provides methods of making an isolated hybridoma which produces an antibody useful for diagnosing a patient or animal with SLE/LN. In this method, a MDK or its variant is isolated (*e.g.*, by purification from a cell in which it is expressed or by transcription and translation of a polynucleotide encoding the protein *in vivo* or *in vitro* using known methods). A vertebrate, preferably a mammal such as a mouse, rabbit or sheep, is immunized using the isolated polypeptide or polypeptide fragment. The vertebrate may optionally (and preferably) be immunized at least one additional time with the isolated polypeptide or polypeptide fragment, so that the vertebrate exhibits a robust immune response to the polypeptide or polypeptide fragment. Splenocytes are isolated from the immunized vertebrate and fused with an immortalized cell line to form hybridomas, using any of a variety of methods well known in the art. Hybridomas formed in this manner are then screened using standard methods to identify one or more hybridomas which produce an antibody which specifically binds with the polypeptide or polypeptide fragment. The invention also includes hybridomas made by this method and antibodies made using such hybridomas.

**[0150]** An isolated MDK polypeptide, or a portion or fragment thereof, can be used as an immunogen to generate antibodies that bind the MDK polypeptide using standard techniques for polyclonal and monoclonal antibody preparation. A full-length MDK polypeptide can be used or, alternatively, the invention provides antigenic peptide fragments of the MDK polypeptide for use as immunogens. The antigenic peptide of a MDK polypeptide preferably comprises at least 8 amino acid residues of an amino acid sequence encoded by a MDK gene, and encompasses an epitope of a MDK polypeptide such that an antibody raised against the peptide forms a specific immune complex with the MDK polypeptide. Preferably, the antigenic peptide comprises at least 8 amino acid residues, more preferably at least 12 amino acid residues, even more preferably at least 16 amino acid residues, and most preferably at least 20 amino acid residues.

**[0151]** Immunogenic portions (*i.e.*, epitopes) may generally be identified using well known techniques. Such techniques include screening polypeptides for the ability to react with antigen-specific antibodies, antisera and/or T-cell lines or clones. Such antisera and antibodies may be prepared as described herein, and using well known techniques. An

obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique, human B cell hybridoma technique, the EBV-hybridoma technique, or trioma techniques. The technology for producing monoclonal antibody hybridomas is well known. Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with a MDK immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds to MDK.

**[0156]** Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-MDK monoclonal antibody. Moreover, the ordinarily skilled worker will appreciate that there are many variations of such methods which also would be useful. Typically, the immortal cell line (*e.g.*, a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an immortalized mouse cell line. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques, *e.g.*, the P3-NS1/l-Ag4-1, P3-x63-Ag8.653 or Sp210-Ag14 myeloma lines. These myeloma lines are available from ATCC. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody are detected by screening the hybridoma culture supernatants for antibodies that bind to MDK specifically, *e.g.*, using a standard ELISA assay.

**[0157]** Alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal anti-MDK antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (*e.g.*, an antibody phase display library) with MDK to thereby isolate immunoglobulin library members that bind to MDK. Kits for generating and screening phage display libraries are commercially available (*e.g.*, the Pharmacia

immunized in the normal fashion with a selected antigen, *e.g.*, all or a portion of a MDK polypeptide. Monoclonal antibodies directed against the antigen can be obtained using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA and IgE antibodies.

**[0162]** Humanized antibodies which recognize a selected epitope can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, *e.g.*, a murine antibody, is used to guide the selection of a humanized antibody recognizing the same epitope.

**[0163]** In a preferred embodiment, the antibodies to MDK are capable of reducing or eliminating the biological function of MDK. Generally, at least a 25% decrease in activity is preferred, with at least about 50% being particularly preferred and about a 95-100% decrease being especially preferred.

**[0164]** An anti-MDK antibody can be used to isolate MDK by standard techniques, such as affinity chromatography or immunoprecipitation. An anti-MDK antibody can facilitate the purification of natural MDKs from cells and of recombinantly produced MDKs expressed in host cells. Moreover, an anti-MDK antibody can be used to detect MDK (*e.g.*, in a cellular lysate or cell supernatant on the cell surface) in order to evaluate the abundance and pattern of expression of MDK. Anti-MDK antibodies can be used diagnostically to monitor protein levels in tissue as part of a clinical testing procedure, for example, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling (*i.e.*, physically linking) the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin; and examples of suitable radioactive materials include $^{125}I$, $^{131}I$, $^{35}S$ or $^{3}H$.

4,638,045), by serum complement-mediated hydrolysis (e.g., U.S. Pat. No. 4,671,958), and acid-catalyzed hydrolysis (*e.g.*, U.S. Pat. No. 4,569,789).

**[0170]** It may also be desirable to couple more than one agent to an antibody. In one embodiment, agents are coupled to one antibody molecule. In another embodiment, at least two different types of agent may be coupled to one antibody. Regardless of the particular embodiment, immunoconjugates coupled with more than one agent can be prepared in a variety of ways, as appreciated by one or ordinary skill in the art.

***Vectors, Expression Vectors and Gene Delivery Vectors***

**[0171]** Another aspect of the invention pertains to vectors containing a polynucleotide encoding MDK or a portion thereof. One type of vector is a "plasmid," which includes a circular double stranded DNA loop into which additional DNA segments can be ligated. In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of vectors, such as expression vectors, and gene delivery vectors.

**[0172]** The expression vectors of the invention comprise a polynucleotide encoding MDK or a portion thereof in a form suitable for expression of the polynucleotide in a host cell, which means that the expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operatively linked to the polynucleotide sequence to be expressed. It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, and the like. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by polynucleotides as described herein (*e.g.*, MDK, variants of MDK, MDK fusion proteins, and the like).

**[0173]** The expression vectors of the invention can be designed for expression of MDK or its variants in prokaryotic or eukaryotic cells. For example, MDK can be expressed in bacterial cells such as *E. coli*, insect cells (*e.g.*, using baculovirus expression vectors) yeast cells or mammalian cells. In certain embodiments, such protein may be used, for example, as a therapeutic protein of the invention. Alternatively, the expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

[0178] In another embodiment, the MDK expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1, pMFa, pJRY88, pYES2 (Invitrogen Corporation, San Diego, CA), and picZ (Invitrogen Corp, San Diego, CA).

[0179] Alternatively, MDK can be expressed in insect cells using baculovirus expression vectors. Suitable baculovirus vectors available for expression of proteins in cultured insect cells (*e.g.*, Sf9 cells) include the pAc series and the pVL series.

[0180] In yet another embodiment, MDK or its variant is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 and pMT2PC. When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, adenovirus 2, cytomegalovirus and Simian Virus 40. Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac fusion promoter. Target gene expression from the pET 11d vector relies on transcription from a T7 gn10-lac fusion promoter mediated by a coexpressed viral RNA polymerase (T7 gn1). This viral polymerase is supplied by host strains BL21 (DE3) or HSLE174(DE3) from a resident prophage harboring a T7 gn1 gene under the transcriptional control of the lacUV 5 promoter.

[0181] In another embodiment, the mammalian expression vector is capable of directing expression of the polynucleotide preferentially in a particular cell type (*e.g.*, tissue-specific regulatory elements are used to express the polynucleotide). Tissue-specific regulatory elements are known in the art and may include epithelial cell-specific promoters. Examples of suitable tissue-specific promoters include the liver-specific albumin promoter, lymphoid-specific promoters, promoters of T cell receptors and immunoglobulins, neuron-specific promoters (*e.g.*, the neurofilament promoter), pancreas-specific promoters, and mammary gland-specific promoters (*e.g.*, milk whey promoter). Developmentally-regulated promoters are also encompassed, for example the α-fetoprotein promoter.

[0182] The present invention also provides a recombinant expression vector comprising a polynucleotide which encodes MDK but is cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression (*e.g.*, via transcription of the DNA molecule) of an RNA molecule which is antisense to mRNA corresponding to MDK

are efficiently transported into cells after endocytosis initiation by the beads. This method may be improved further by treatment of the beads to increase hydrophobicity and thereby facilitate disruption of the endosome and release of the DNA into the cytoplasm.

***Regulatable Expression Systems***

**[0185]** Another aspect of the invention pertains to the expression of polynucleotides or polypeptides that are capable of inhibiting MDK activity or MDK expression using a regulatable expression system. Systems to regulate expression of therapeutic genes have been developed and incorporated into the current viral and nonviral gene delivery vectors. These systems are briefly described below:

**[0186]** *Tet-on/off system.* The Tet-system is based on two regulatory elements derived from the tetracycline-resistance operon of the E. coli Tn10 transposon: the tet repressor protein (TetR) and the Tet operator DNA sequence (tetO) to which TetR binds. The system consists of two components, a "regulator" and a "reporter" plasmid. The "regulator" plasmid encodes a hybrid protein containing a mutated Tet repressor (rtetR) fused to the VP16 activation domain of herpes simplex virus. The "reporter" plasmid contains a tet-responsive element (TRE), which controls the "reporter" gene of choice. The rtetR-VP16 fusion protein can only bind to the TRE, therefore activating the transcription of the "reporter" gene in the presence of tetracycline. The system has been incorporated into a number of viral vectors including retrovirus, adenovirus and AAV (Gossen *et al.*, Science 268: 1766-1769, 1995).

**[0187]** *Ecdysone system.* The Ecdysone system is based on the molting induction system found in *Drosophila*, but modified for inducible expression in mammalian cells. The system uses an analog of the *Drosophila* steroid hormone ecdysone, muristerone A, to activate expression of the gene of interest via a heterodimeric nuclear receptor. Expression levels have been reported to exceed 200-fold over basal levels with no effect on mammalian cell physiology (No *et al.*, Proc. Natl. Acad. Sci. USA 93: 3346-3351, 1996).

**[0188]** *Progesterone-system.* The progesterone receptor is normally stimulated to bind to a specific DNA sequence and to activate transcription through an interaction with its hormone ligand. Conversely, the progesterone antagonist mifepristone (RU486) is able to block hormone-induced nuclear transport and subsequent DNA binding. A mutant form of the progesterone receptor that can be stimulated to bind through an interaction with

[0192] Typical methodologies for peptide detection include protein extraction from a cell or tissue sample, followed by binding of an antibody specific for the target protein to the protein sample, and detection of the antibody. For example, detection of midkine may be accomplished using polyclonal anti-midkine antibody. Antibodies are generally detected by the use of a labeled secondary antibody. The label can be a radioisotope, a fluorescent compound, an enzyme, an enzyme co-factor, or ligand. Such methods are well understood in the art.

[0193] In certain embodiments, the MDK gene itself (*i.e.*, the DNA or cDNA) may serve as a marker for SLE/LN. For example, an increase of genomic copies of a MDK gene, such as by duplication of the gene, may be correlated with SLE/LN.

[0194] Detection of specific polynucleotides may also be assessed by gel electrophoresis, column chromatography, or direct sequencing, quantitative PCR (in the case of polynucleotide), RT-PCR, or nested-PCR among many other techniques well known to those skilled in the art.

[0195] Detection of the presence or number of copies of all or a part of a MDK gene may be performed using any method known in the art. Typically, it is convenient to assess the presence and/or quantity of a DNA or cDNA by Southern analysis, in which total DNA from a cell or tissue sample is extracted, is hybridized with a labeled probe (*i.e.*, a complementary DNA molecules), and the probe is detected. The label group can be a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. Other useful methods of DNA detection and/or quantification include direct sequencing, gel electrophoresis, column chromatography, and quantitative PCR, as is known by one skilled in the art.

***Screening Methods***

[0196] The present invention also provides methods (also referred to herein as "screening assays") for identifying modulators, *i.e.*, candidate or test compounds or agents comprising therapeutic moieties (*e.g.*, peptides, peptidomimetics, peptoids, polynucleotides, small molecules or other drugs) which (a) bind to MDK, or (b) have a modulatory (*e.g.*, stimulatory or inhibitory) effect on the activity of MDK or, more specifically, (c) have a modulatory effect on the interactions of MDK with one or more of its natural substrates (*e.g.*, peptide, protein, hormone, co-factor, or polynucleotide), or (d) have a modulatory effect on the expression of MDK. Such assays typically comprise a

diagnosed with or suspected of having SLE/LN, contacting each separate aliquot of the samples with one of a plurality of test compounds, and comparing expression of MDK in each of the aliquots to determine whether any of the test compounds provides a substantially decreased level of expression or activity of MDK relative to samples with other test compounds or relative to an untreated sample or control sample. In addition, methods of screening may be devised by combining a test compound with a protein and thereby determining the effect of the test compound on the protein.

**[0201]** In addition, the invention is further directed to a method of screening for test compounds capable of modulating with the binding of MDK and a binding partner, by combining the test compound, MDK, and binding partner together and determining whether binding of the binding partner and MDK occurs. The test compound may be either small molecules or a bioactive agent. As discussed below, test compounds may be provided from a variety of libraries well known in the art.

**[0202]** Inhibitors of MDK expression, activity or binding ability are useful as therapeutic compositions of the invention. Such inhibitors may be formulated as pharmaceutical compositions, as described herein below. Such modulators may also be used in the methods of the invention, for example, to diagnose, treat, or prognose SLE/LN.

***High-Throughput Screening Assays***

**[0203]** The present invention also provides methods for conducting high-throughput screening for test compounds capable of inhibiting activity or expression of MDK. In one embodiment, the high-throughput screening method involves contacting test compounds with MDK and then detecting the effect of the test compounds on MDK. Functional assays such as cytosensor microphysiometer-based assays, calcium flux assays such as FLIPR® (Molecular Devices Corp, Sunnyvale, CA), or the TUNEL assay may be employed to measure cellular activity, as discussed below.

**[0204]** A variety of high-throughput functional assays well-known in the art may be used in combination to screen and/or study the reactivity of different types of activating test compounds. Since the coupling system is often difficult to predict, a number of assays may need to be configured to detect a wide range of coupling mechanisms. Fluoroescence–based techniques are well-known in the art and are capable of high-throughput and ultra high throughput screening. They include, but are not limited to BRET® and FRET® (both by Packard Instrument Co., Meriden, CT). The ability to screen

*Diagnostic Assays*

[0207] An exemplary method for detecting the presence of MDK or polynucleotide encoding MDK in a biological sample involves obtaining a biological sample from a test subject and contacting the biological sample with a compound or an agent capable of detecting the protein or polynucleotide (*e.g.*, mRNA, genomic DNA) that encodes MDK such that the presence of MDK or polynucleotide is detected in the biological sample. A preferred agent for detecting mRNA or genomic DNA corresponding to a MDK gene or MDK protein is a labeled polynucleotide probe capable of hybridizing to a MDK mRNA or a genomic DNA. Suitable probes for use in the diagnostic assays of the invention are described herein. A preferred agent for detecting MDK is a MDK-specific antibody which specifically recognizes MDK.

[0208] The diagnostic assays may also be used to quantify the amount of expression or activity of MDK in a biological sample. Such quantification is useful, for example, to determine the progression or severity of SLE/LN. Such quantification is also useful, for example, to determine the severity of SLE/LN following treatment.

*Determining severity of SLE/LN*

[0209] In the field of diagnostic assays, the invention also provides methods for determining the severity of SLE/LN by isolating a sample from a subject, detecting the presence, quantity and/or activity of MDK in the sample relative to a second sample from a normal sample or control sample. In one embodiment, the expression levels of MDK in the two samples are compared, and an increased MDK expression in the test sample indicates SLE/LN.

[0210] A preferred agent for detecting MDK is an antibody capable of binding to MDK, preferably an antibody with a detectable label. Antibodies can be polyclonal or more preferably, monoclonal. An intact antibody, or a fragment thereof (*e.g.*, Fab or $F(ab')_2$) can be used. The term "labeled," with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (*i.e.*, physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with

activity is diagnostic for a subject that can be administered the agent to treat injury associated with aberrant MDK expression or activity).

**[0214]** Prognostic assays can be devised to determine whether a subject undergoing treatment for SLE/LN has a poor outlook for long term survival or disease progression. In a preferred embodiment, prognosis can be determined shortly after diagnosis, *i.e.*, within a few days. By establishing MDK expression profiles of different stages of SLE/LN, from onset to later stages, an expression pattern may emerge to correlate a particular expression profile to increased likelihood of a poor prognosis. The prognosis may then be used to devise a more aggressive treatment program and enhance the likelihood of long-term survival and well being.

**[0215]** The methods of the invention can also be used to detect genetic alterations in a MDK gene, thereby determining if a subject with the altered gene is at risk for damage characterized by aberrant regulation in MDK activity or polynucleotide expression. In preferred embodiments, the methods include detecting, in a sample of cells from the subject, the presence or absence of a genetic alteration characterized by at least one alteration affecting the integrity of a MDK gene, or the aberrant expression of the MDK gene. For example, such genetic alterations can be detected by ascertaining the existence of at least one of the following: 1) deletion of one or more nucleotides from a MDK gene; 2) addition of one or more nucleotides to a MDK gene; 3) substitution of one or more nucleotides of a MDK gene, 4) a chromosomal rearrangement of a MDK gene; 5) alteration in the level of a messenger RNA transcript of a MDK gene, 6) aberrant modification of a MDK gene, such as of the methylation pattern of the genomic DNA, 7) the presence of a non-wild type splicing pattern of a messenger RNA transcript of a MDK gene, 8) non-wild type level MDK, 9) allelic loss of a MDK gene, and 10) inappropriate post-translational modification of MDK. As described herein, there are a large number of assays known in the art, which can be used for detecting alterations in a MDK gene. A preferred biological sample is a blood sample isolated by conventional means from a subject.

**[0216]** In certain embodiments, detection of the alteration involves the use of a probe/primer in a polymerase chain reaction (PCR), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR), the latter of which can be particularly useful for detecting point mutations in the MDK gene. This method can include the steps of collecting a sample of cells from a subject, isolating polynucleotide (*e.g.*, genomic,

composed of parallel probe sets, one complementary to the wild-type gene and the other complementary to the mutant gene.

**[0220]** In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence the MDK gene and detect mutations by comparing the sequence of the sample MDK gene with the corresponding wild-type (control) sequence. It is also contemplated that any of a variety of automated sequencing procedures can be utilized when performing the diagnostic assays, including sequencing by mass spectrometry.

**[0221]** Other methods for detecting mutations in a MDK gene include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA heteroduplexes (Myers *et al., Science* 230:1242, 1985). In general, "mismatch cleavage" technique involves forming heteroduplexes by hybridizing a RNA or DNA (labeled) containing the wild-type MDK gene sequence to a potentially mutant RNA or DNA obtained from a tissue sample. The double-stranded duplexes are treated with an agent which cleaves single-stranded regions of the duplex which will exist due to basepair mismatches between the control and sample strands. For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA hybrids treated with S1 nuclease to enzymatically digest the mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched regions, the resulting material is then separated by size on denaturing polyacrylamide gels to determine the site of mutation. In a preferred embodiment, the control DNA or RNA can be labeled for detection.

**[0222]** In still another embodiment, the mismatch cleavage reaction employs one or more proteins that recognize mismatched base pairs in double-stranded DNA (so called "DNA mismatch repair" enzymes) in defined systems for detecting and mapping point mutations in MDK cDNAs obtained from samples of cells. For example, the mutY enzyme of *E. coli* cleaves A at G/A mismatches and the thymidine DNA glycosylase from HeLa cells cleaves T at G/T mismatches. According to an exemplary embodiment, a probe based on a MDK gene sequence, *e.g.*, a wild-type MDK gene sequence, is hybridized to cDNA or other DNA product from a test cell(s). The duplex thus formed is treated with a DNA mismatch repair enzyme, and the cleavage products, if any, can be detected from electrophoresis protocols or the like. *See e.g.*, U.S. Patent No. 5,459,039.

hybridization) or at the extreme 3' end of one primer where, under appropriate conditions, mismatch can prevent or reduce polymerase extension. See, for example, Saiki *et al.*, Proc. Natl. Acad. Sci USA 86:6230, 1989) In addition it may be desirable to introduce a novel restriction site in the region of the mutation to create cleavage-based detection. It is anticipated that in certain embodiments amplification may also be performed using Taq ligase for amplification. In such cases, ligation will occur only if there is a perfect match at the 3' end of the 5' sequence making it possible to detect the presence of a known mutation at a specific site by looking for the presence or absence of amplification.

**[0227]** The methods described herein may be performed, for example, by using prepackaged diagnostic kits comprising at least one polynucleotide probe or one antibody of the present invention. These kits can be in clinical settings to diagnose subjects exhibiting symptoms or family history of SLE/LN. Furthermore, any cell type or tissue in which MDK is expressed may be used in the prognostic or diagnostic assays described herein.

***Monitoring Effects During Clinical Trials***

**[0228]** Monitoring the influence of agents (*e.g.*, drugs, small molecules, proteins, nucleotides) on the expression or activity of MDK can be applied not only in basic drug screening, but also in clinical trials. For example, the effectiveness of an agent determined by a screening assay, as described herein to decrease MDK expression, protein levels, or downregulate MDK activity, can be monitored in clinical trials of subjects exhibiting increased MDK expression, protein levels, or upregulated MDK activity. In such clinical trials, the expression or activity of MDK can be used as a "read out" of the phenotype of a particular tissue.

**[0229]** For example, to study the effect of agents on MDK-associated damage in a clinical trial, cells can be isolated and RNA prepared and analyzed for the levels of expression of MDK. The levels of gene expression can be quantified by northern blot analysis, RT-PCR, GeneChip® or Taqman analysis as described herein, or alternatively by measuring the amount of protein produced, by one of the methods as described herein, or by measuring the levels of activity of MDK. In this way, the gene expression level can serve as a read-out, indicative of the physiological response of the cells to the agent. Accordingly, this response state may be determined before treatment and at various points during treatment of the individual with the agent.

*Prophylactic Methods*

[0232] The present invention further provides a method for preventing in a subject SLE/LN associated with aberrant MDK expression or activity, by administering to the subject an agent which modulates MDK protein expression or activity.

[0233] Subjects at risk for SLE/LN which is caused or contributed to by aberrant MDK expression or activity can be identified by, for example, any or a combination of diagnostic or prognostic assays as described herein.

[0234] Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the differential MDK protein expression, such that SLE/LN is prevented or, alternatively, delayed in its progression. Depending on the type of MDK aberrancy (*e.g.*, typically a modulation outside the normal standard deviation), for example, a MDK mutant protein, MDK antagonist agent, or MDK antisense polynucleotide can be used for treating the subject. The appropriate agent can be determined based on screening assays described herein.

*Therapeutic Methods*

[0235] Another aspect of the invention pertains to methods of modulating MDK protein expression or activity for therapeutic purposes. Accordingly, in an exemplary embodiment, the modulatory method of the invention involves contacting a cell with an agent that inhibits MDK gene expression or one or more of the activities of MDK protein associated with the cell. An agent that modulates MDK gene expression or protein activity can be an agent as described herein, such as a polynucleotide, a polypeptide, or a polysaccharide, a naturally-occurring target molecule of a MDK protein (*e.g.*, a MDK protein substrate or receptor), an anti-MDK antibody, a MDK antagonist, a peptidomimetic of a MDK antagonist, or other small organic and inorganic molecule.

[0236] These modulatory methods can be performed *in vivo* (*e.g.*, by administering the agent to a subject). As such, the present invention provides methods of treating an individual diagnosed with or at risk for SLE/LN characterized by aberrant expression or activity of MDK. In one embodiment, the method involves administering an agent (*e.g.*, an agent identified by a screening assay described herein), or combination of agents that downregulates MDK expression or activity. The agent may include a vector comprising a polynucleotide encoding a MDK inhibitor or an antisense sequence. The agent may be an anti-MDK antibody, a plurality of anti-MDK antibodies or an anti-MDK antibody

polymorphisms (SNPs) in the human genome. A "SNP" is a common alteration that occurs in a single nucleotide base in a stretch of DNA. For example, a SNP may occur once per every 1000 bases of DNA. A SNP may be involved in a disease process. However, the vast majority of SNPs may not be disease associated. Given a genetic map based on the occurrence of such SNPs, individuals can be grouped into genetic categories depending on a particular pattern of SNPs in their individual genome. In such a manner, treatment regimens can be tailored to groups of genetically similar individuals, taking into account traits that may be common among such genetically similar individuals. Thus, mapping of the MDK gene to SNP maps of LN patients may allow easier identification of these genes according to the genetic methods described herein.

**[0240]** Alternatively, a method termed the "candidate gene approach," can be utilized to identify genes that predict drug response. According to this method, if a gene that encodes a drug target is known (*e.g.*, MDK), all common variants of that gene can be fairly easily identified in the population. It then can be determined if a particular drug response is associated with one version of the gene versus another is associated with a particular drug response.

**[0241]** The activity of drug metabolizing enzymes is a major determinant of both the intensity and duration of drug action. The discovery of genetic polymorphisms of drug metabolizing enzymes (*e.g.*, N-acetyltransferase 2 (NAT 2) and cytochrome P450 enzymes CYP2D6 and CYPZC19) has provided an explanation as to why some subjects do not obtain the expected drug effects or show exaggerated drug response and serious toxicity after taking the standard and safe dose of a drug. These polymorphisms are expressed in two phenotypes in the population, the extensive metabolizer and poor metabolizer. The prevalence of poor metabolizer phenotypes is different among different populations. For example, the gene coding for CYP2D6 is highly polymorphic and several mutations have been identified in poor metabolizers, which all lead to the absence of functional CYP2D6. Poor metabolizers of CYP2D6 and CYP2C19 quite frequently experience exaggerated drug response and side effects when they receive standard doses. If a metabolite is the active therapeutic moiety, poor metabolizers show no therapeutic response, as demonstrated for the analgesic effect of codeine mediated by its CYP2D6-formed metabolite morphine. The other extreme are the so called ultra-rapid metabolizers who do not respond to standard doses. Recently, the molecular basis of ultra-rapid metabolism has been identified to be due to CYP2D6 gene amplification.

chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

**[0247]** Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the injectable composition should be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

**[0248]** Sterile injectable solutions can be prepared by incorporating the active modulator (*e.g.*, an anti-MDK antibody, a MDK activity inhibitor, or a gene therapy vector expressing antisense nucleotide to MDK) in the required amount in an appropriate solvent, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

apparent to those skilled in the art. The materials can also be obtained commercially from, *e.g.*, Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

**[0254]** It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein includes physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

**[0255]** Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

**[0256]** The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell

host cell's genome. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

**[0262]** A host cell can be any prokaryotic or eukaryotic cell. For example, a MDK gene can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (*e.g.*, Chinese hamster ovary cells (CHO), COS cells, Fischer 344 rat cells, HLA-B27 rat cells, HeLa cells, A549 cells, or 293 cells). Other suitable host cells are known to those skilled in the art.

**[0263]** Vector DNA can be introduced into prokaryotic or eukaryotic cells *via* conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign polynucleotide (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DAKD-dextran-mediated transfection, lipofection, or electoporation.

**[0264]** For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable flag (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable flags include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Polynucleotides encoding a selectable flag can be introduced into a host cell by the same vector as that encoding MDK or can be introduced by a separate vector. Cells stably transfected with the introduced polynucleotide can be identified by drug selection (*e.g.*, cells that have incorporated the selectable flag gene will survive, while the other cells die).

**[0265]** A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (*i.e.*, express) MDK. Accordingly, the invention further provides methods for producing MDK using the host cells of the invention. In one embodiment, the method comprises culturing the host cell of invention (into which a recombinant expression vector containing a MDK gene has been introduced) in a suitable

animals. A transgenic founder animal can be identified based upon the presence of a transgene of the invention in its genome and/or expression of mRNA corresponding to a gene of the invention in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene encoding MDK can further be bred to other transgenic animals carrying other transgenes.

**[0268]** To create a homologous recombinant animal (knockout animal), a vector is prepared which contains at least a portion of a gene of the invention into which a deletion, addition or substitution has been introduced to thereby alter, *e.g.*, functionally disrupt, the gene. The gene can be a human gene, but more preferably, is a non-human homolog of a human gene of the invention (*e.g.*, a homolog of the MDK gene). For example, a mouse gene can be used to construct a homologous recombination polynucleotide, *e.g.*, a vector, suitable for altering an endogenous gene of the invention in the mouse genome. In a preferred embodiment, the homologous recombination polynucleotide is designed such that, upon homologous recombination, the endogenous gene of the invention is functionally disrupted (*i.e.*, no longer encodes a functional protein; also referred to as a "knockout" vector). Alternatively, the homologous recombination polynucleotide can be designed such that, upon homologous recombination, the endogenous gene is mutated or otherwise altered but still encodes functional protein (*e.g.*, the upstream regulatory region can be altered to thereby alter the expression of the endogenous MDK gene). In the homologous recombination polynucleotide, the altered portion of the gene of the invention is flanked at its 5' and 3' ends by additional polynucleotide sequence of the gene of the invention to allow for homologous recombination to occur between the exogenous gene carried by the homologous recombination polynucleotide and an endogenous gene in a cell, *e.g.*, an embryonic stem cell. The additional flanking polynucleotide sequence is of sufficient length for successful homologous recombination with the endogenous gene.

**[0269]** Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the homologous recombination polynucleotide. The homologous recombination polynucleotide is introduced into embryonic stem cells by electroporation. The cells in which the introduced gene has homologously recombined with the endogenous gene are selected. The selected cells can then be injected into a blastocyst of an animal (*e.g.*, a mouse) to form aggregation chimeras. A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to

The rapamycin-treated NZBxNZW F1 mice was injected with rapamycin subcutaneously, 5mg/kg, 3 times per week for 8 weeks, with treatment beginning at 29 weeks of age.

**[0273]** Kidneys from both male and female mice were collected and snap frozen for RNA isolation. One half of each kidney (a longitudinal section of the left kidney and a cross section of the right kidney) was harvested from each mouse in the study. Snap frozen mouse kidney tissue was homogenized using homogenizer suspended in RLT buffer plus 2-mercaptoethanol for 30 to 45 seconds. Total RNA was prepared using the Qiagen Midi Kit following the manufacturer's protocol. RNA was suspended in DEPC-treated water and quantified by OD 280.

**[0274]** Gene expression analysis was performed on individual kidney RNA samples harvested from the following mice: C57BL/6 female mice at 8 weeks (n=3), 20 weeks (n=3) and 32 (n=3) weeks; MRL/MpJ-*Fas*$^{lpr}$ male at 8 weeks (n=3) and 20 weeks (n=2); MRL/MpJ-*Fas*$^{lpr}$ female mice at 8 weeks (n=3), 16 weeks and 20 weeks (n=6 combined), MRL/MpJ female mice at 8 (n=3) and 20 weeks (n=3), MRL/MpJ male mice at 8 (n=3) and 24 weeks (n=2), B6/MRL-*Fas*$^{lpr}$ male at 8 weeks (n=3) and 20 weeks (n=3) and B6/MRL-*Fas*$^{lpr}$ female mice at 8 weeks (n=3) and 20 weeks (n=3). Thus the total number of individual RNA samples subjected to gene expression analysis using the Affymetrix Gene chip arrays was 46, 21 of which were harvested from lupus nephritis-free stains and the remainder from mice either before, during or after disease onset.

**[0275]** cDNA was synthesized from 5μg of total RNA from each individual kidney sample using the Superscript Kit (Life Technologies, Rockville, MD) with modifications described in detail previously Byrne *et al.* (Byrne, *et al.*, in Current Protocols in Molecular Biology, John Wiley and Sons, Inc, New York, 2000). cDNA was purified using phenol:cloroform:isoamyl alcohol (25:24:1) with a Phage lock gel tube following the Phage lock protocol. Supernatant was collected and cleaned up using ethanol. Sample was resuspended in DEPC-treated water.

**[0276]** *In vitro* T7 polymerase driven transcription reactions for synthesis and biotin labeling of antisense cRNA, Qiagen Rneasy spin column purification and cRNA fragmentation were carried out in as previously described (Lockhart *et al.*, Nature Biotechnology 14, 1675-80, 1996). GeneChip hybridization mixtures contained 15μg fragmented cRNA, 0.5mg/ml acetylated BSA, 0.1mg/ml herring sperm DNA, in 1X MES buffer in a total volume of 200μl as per manufactures instructions. Reaction mixtures were hybridized for 16hr at 45°C to Affymetrix Mu11KsubA and Mu11KsubB

viewed as estimates, and inter-gene comparisons of frequencies should be interpreted cautiously.

*Example 3: Selection of Genes in Analysis Set:*

**[0278]** The detection of any gene was deemed unreliable if it was not called present in at least 50% of samples from at least one group and was eliminated from the set of genes under analysis. Similarly, in order to avoid conclusions dependent on the lower (and less reliable by Taqman PCR) limits of the standard curve, any gene with average frequency not greater than 9 ppm in at least one group was eliminated from analysis. These operations resulted in a list of 5,285 tiled oligonucleotides representing the set of genes to be surveyed for MRL strain-dependent gene expression differences.

*Example 4: Flagging of Potential Age, Gender and $fas^{lpr}$ Dependent Gene Expression Differences*

**[0279]** Average fold change (AFC) was obtained by dividing the average frequency of one group by the average of the other group. To identify genes whose expression levels are influenced by gender, the AFC between male and female groups was calculated for each of the six groups of male and female mice listed above. All genes with fold change differences consistent between male and female mice in each group combination were flagged as demonstrating a possible gender-influenced. Genes with AFC>1.5 between 8 and 32 week old C57BL/6 (disease free) were flagged as "possibly age-influenced". Gene with AFC >1.5 between C57BL/6 and C57BL/6-$Fas^{lpr}$ were flagged as demonstrating an effect of the $Fas^{lpr}$ mutation that did not depend on the disease prone MRL genetic background. Genes identified through these processes as demonstrating possible gender, age and $Fas^{lpr}$ influences on expression frequency were flagged but retained on the list of genes surveyed for influences related to the MRL genetic background.

*Example 5: Quantitative Reverse Transcriptase-polymerase Chain Reaction (Taqman Analysis)*

**[0280]** Quantitative RT-PCR was performed using RNA samples from murine kidneys were treated with 10U of RQ1 DNase I (Promega, Madison, WI, USA) for 30 minutes at 37°C. 10ng of total RNA was reverse transcribed and amplified in a single tube assay using the TaqMan® One Step PCR Master Mix Reagent Kit (Applied BioSystems,

What is claimed is:

1. A method comprising the steps of:

detecting an expression level of midkine gene in a biological sample isolated from a mammal of interest; and

comparing the expression level to a reference expression level of said midkine gene in at least one control sample.

2. The method of claim 1, wherein said at least one contro sample is isolated from at least one contro mammal, wherein said at least one control mammal does not have systemic lupus erythematosus or lupus nephritis.

3. The method of claim 2, wherein the mammal of interest has systemic lupus erythematosus or lupus nephritis.

4. The method of claim 2, wherein the expression level and the reference expression level are detected using an antibody directed against a product of said midkine gene.

5. The method of claim 2, wherein the expression level and the reference expression level are detected by measuring the level of an RNA transcript of said midkine gene.

6. The method of claim 2, wherein the biological sample is selected from the group consisting of a tissue sample, a urine sample, and a blood sample.

7. The method of claim 2, wherein the biological sample and said at least one control sample are kidney samples.

8. The method of claim 2, wherein the mammal of interest is a human.

9. A pharmaceutical composition for preventing or treating systemic lupus erythematosus or lupus nephritis, comprising a pharmaceutically acceptable carrier and an agent that modulates a midkine activity or midkine gene expression.

10. The pharmaceutical composition of claim 9, wherein the agent inhibits said midkine activity or midkine gene expression.

11. The method of claim 9, wherein the agent is selected from the group consisting of a polypeptide, a polynucleotide, a polysaccharide, a small organic molecule, and an inorganic molecule.

12. The pharmaceutical composition of claim 11, wherein the agent is an antibody that binds specifically to a midkine gene product.

13. The pharmaceutical composition of claim 11, wherein the agent is an antisense polynucleotide to midkine gene.

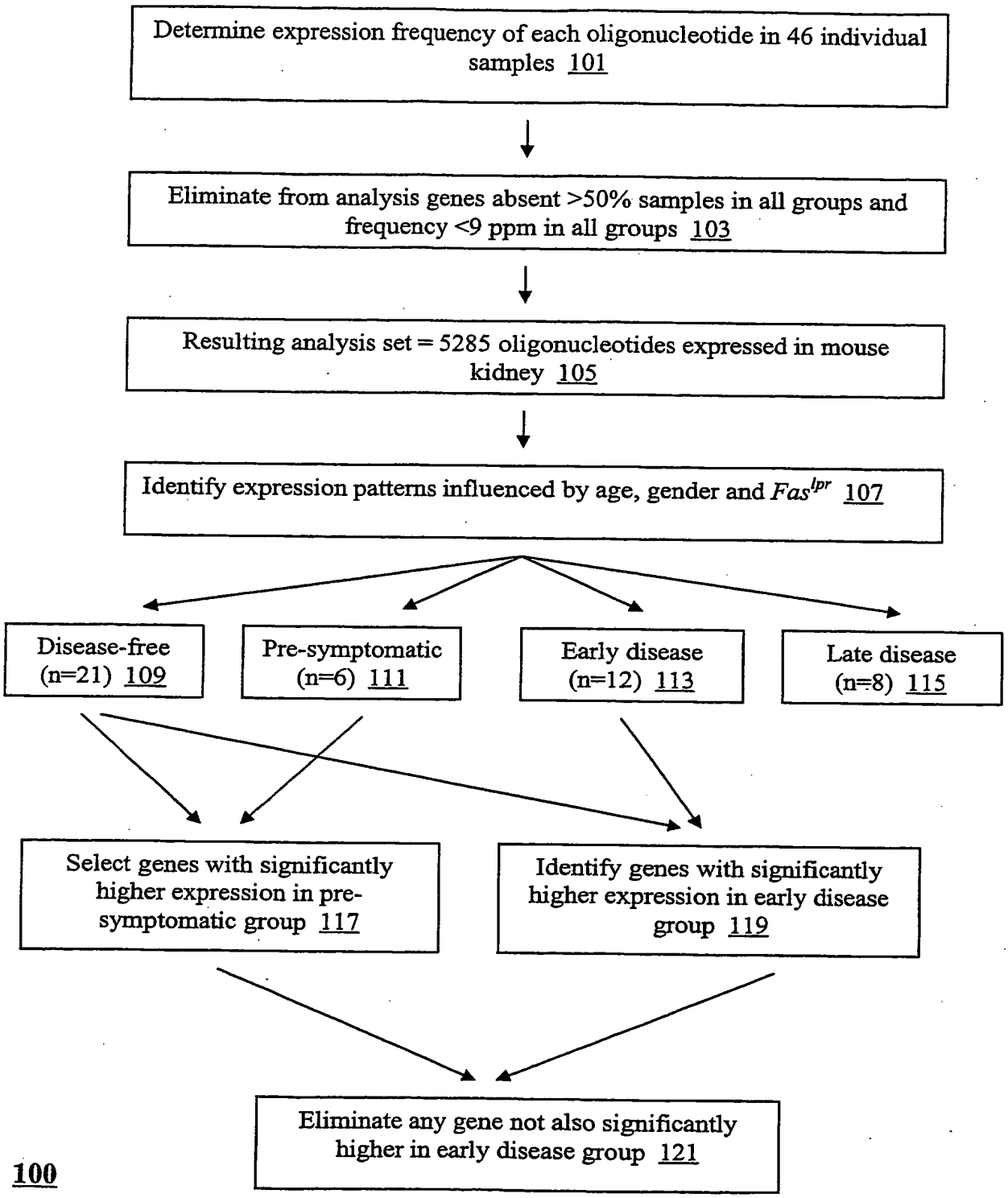
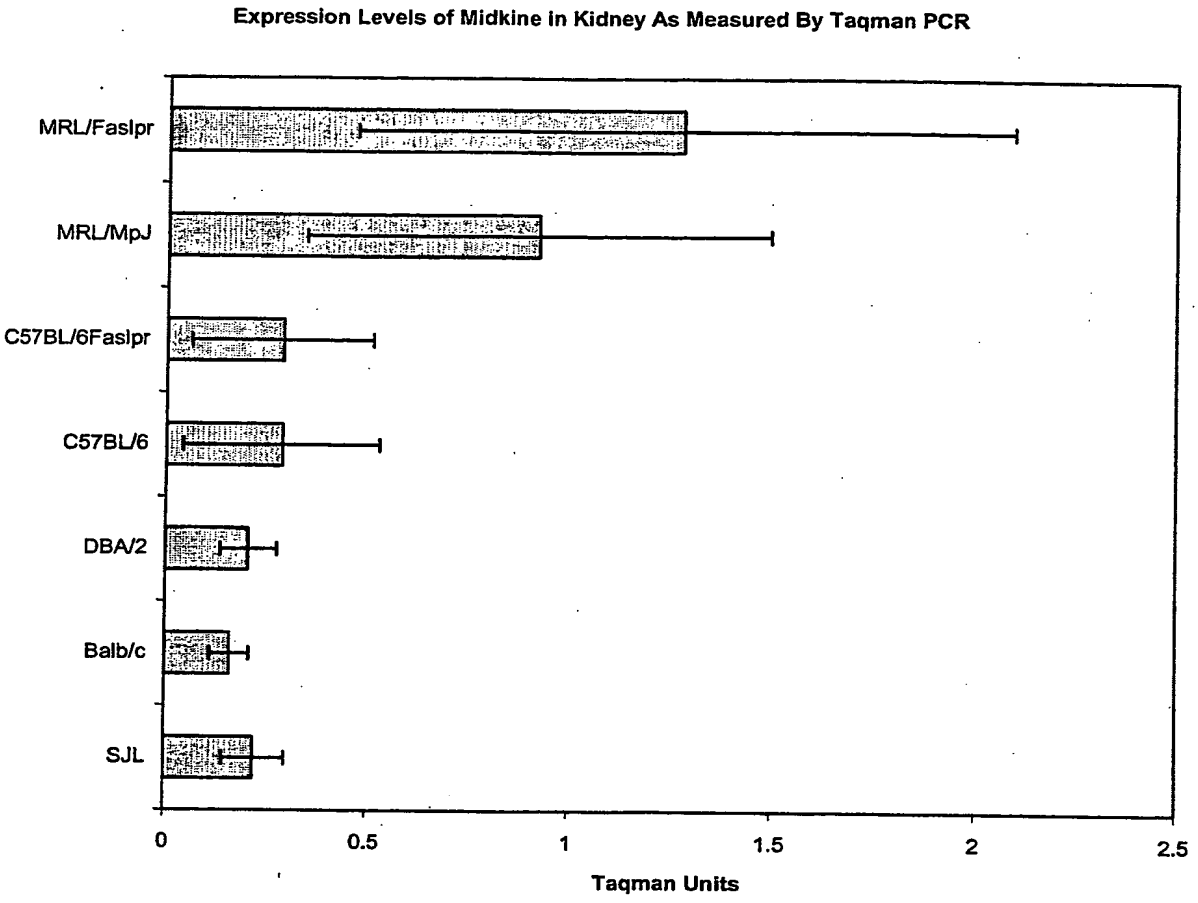

Determine expression frequency of each oligonucleotide in 46 individual samples 101
Eliminate from analysis genes absent >50% samples in all groups and frequency <9 ppm in all groups 103
Resulting analysis set = 5285 oligonucleotides expressed in mouse kidney 105
Identify expression patterns influenced by age, gender and Fas^lpr 107
Disease-free (n=21) 109
Pre-symptomatic (n=6) 111
Early disease (n=12) 113
Late disease (n=8) 115
Select genes with significantly higher expression in pre-symptomatic group 117
Identify genes with significantly higher expression in early disease group 119
Eliminate any gene not also significantly higher in early disease group 121
100

FIGURE 1


Expression Levels of Midkine in Kidney As Measured By Taqman PCR
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MRL/MpJ
C57BL/6Faslpr
C57BL/6
DBA/2
Balb/c
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1.5
2
2.5
Taqman Units

# FIGURE 3

AM100990 Non-Provisonal Seqs.ST25
SEQUENCE LISTING

<110> Wyeth
O'Toole, Margot
Liu, Wei

<120> COMPOSITIONS AND METHODS FOR DIAGNOSING AND TREATING AUTOIMMUNE DISEASE

<130> AM100990

<150> 60/419,088
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Thr Gln Arg Ile Arg Cys Arg Val Pro Cys Asn Trp Lys Lys Glu Phe
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